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14. ABSTRACT

HER-2/neu is a 185 kDa transmembrane protein that is a member of the epidermal growth factor family of receptors and is over expressed on 25-40% of all breast cancers. Five Listeria monocytogenes vaccines have been made consisting of fragments of HER-2/neu that are capable of stopping the growth of transplantable tumors in wild type FVB/N mice and can cause the eventual regression of about 30% of these tumors. Four of the vaccines contain no known epitopes, yet each of the vaccines can lead to anti-HER-2/neu responses. Based on this we began mapping epitopes through cytotoxic T lymphocyte analyses. From this, we have identified a novel epitope that falls into a different region of HER-2/neu than the previously identified epitope. We are studying these epitopes to see if there are similar levels of anti-tumor responses to both of these epitopes. In mice transgenic for rat HER-2/neu these vaccines cause a slowing in the growth of implanted NT-2tumors versus control mice. Regression is not seen in these mice because all of the Lm-LLO-HER-2/neu vaccinated mice scratch away their tumors. An autochthonous tumor experiment shows a much different result, with the vaccines not all behaving identically, but leading to different levels of protection. In this case, Lm-LLO-EC3 does not delay the onset of tumor growth, while each of the other four vaccines does, with Lm-LLO-IC1 being significantly better than all of the other vaccines. We are currently attempting to determine if stromal elements in the mammary gland are involved in this difference.

15. SUBJECT TERMS
HER-2/neu, vaccine, tumor, Listeria monocytogenes

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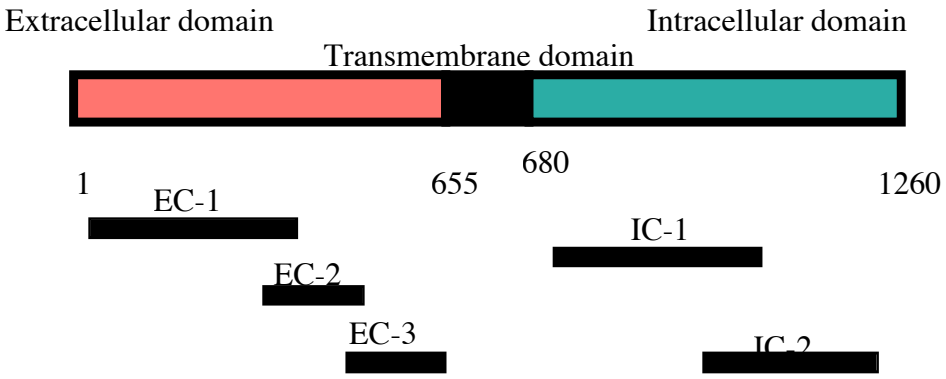
I. Introduction

A promising alternative immunotherapy to the current breast cancer therapies of surgery, radiation, chemotherapy, and hormone replacement, is to target tumor associated antigens (TAAs). This is an attractive alternative due to the fact that they specifically target tumor cells. Over 44,000 women fail one or more of these therapies each year and according to the American Cancer Society, breast cancer has remained the second leading cause of cancer deaths among women in the United States through 2005. In addition to this, over 1500 men are diagnosed with breast cancer each year in the United States alone. 20-40% of all breast cancers overexpress the tumor associated antigen, HER-2/neu, and it is also overexpressed in cancers of the ovaries, lung, pancreas, and gastrointestinal tract (Disis et al., 1997; Knutson et al., 1999; Li et al., 1994). The overexpression of HER-2/neu in breast adenocarcinomas has been associated with the progression of focal tumors to metastatic cancer and the subsequent poor prognosis of patients (Knutson et al., 1999; Treurniet et al., 1992). Despite this, patients with cancers that overexpress HER-2/neu have humoral (Coronella, et al., 2001; Disis et al., 1997b; Disis et al., 2000), $CD8^+$ T cell (peoples et al., 1995), and $CD4^+$ T cell (Tuttle et al., 1998) immune responses directed towards HER-2/neu, but these responses are not effective. Intracellular bacteria, such as *Listeria monocytogenes*, can be used as vaccine vectors due to their ability to generate strong and specific T cell responses (Pan et al., 1995; Pan et al., 1995b; Pan et al., 1999; Pardoll, 1996; Weiskirch et al., 1997). *Listeria* preferentially infects macrophages and other antigen presenting cells, and while most of the bacteria are killed in the phagolysosome, about 10 percent escape into the cytosol. This results in the presentation of listerial antigens by both the MHC class I and class II pathways leading to the subsequent activation of both $CD8^+$ and $CD4^+$ T cells (Hsieh et al., 1993; Weiskirch et al., 2001). Our lab has previously shown that *Listeria monocytogenes* expressing tumor-associated antigens can induce the regression of established tumors that express those antigens (Gunn et al, 2001) and we have adapted this system for the breast cancer associated antigen HER-2/neu. We have previously reported the production of five *Listeria* based vaccines consisting of fragments of HER-2/neu and that these vaccines are all capable of impacting on tumor growth (Singh et al., 2005). After vaccination of tumor bearing mice with the Lm-LLO-HER-2/neu vaccines, the tumors stop growing and remain stable for over 90 days with a small number (about 30%) undergoing complete tumor regression (Singh et al., 2005). Following this we conducted an analysis of the $CD8^+$ T cell pools that are stimulated in these mice through CTL analysis and reported in the previous year that through this method we had identified regions of HER-2/neu that potentially contain unidentified $CD8^+$ T cell epitopes. Further, we have demonstrated that transplanted tumors into FVB/N HER-2/neu transgenic mice grow at a significantly slower rate in Lm-LLO-HER-2/neu vaccinated mice as compared to control mice. In this past year, we have continued studying these vaccines in the transgenic mice and further studied the $CD8^+$ T cell responses in these mice.

II. Body

This study is aimed at determining the effectiveness of *Listeria monocytogenes* based immunotherapies for HER-2/neu overexpressing breast cancers and the mechanisms involved in priming an immune response upon vaccination. *Listeria monocytogenes* that express fragments of HER-2/neu prime $CD8^+$ and may prime $CD4^+$ T cell response that are robust enough to overcome tolerance in a transgenic mouse model of HER-2/neu overexpressing breast cancer. Previous studies in our lab have shown that *Listeria monocytogenes* that express the viral protein E7 are capable of eliciting a strong enough anti-tumor response to E7 to cause the regression of tumors in mice (Gunn et al., 2001). Furthermore, after adapting this system to the self-protein HER-2/neu we show that a potent anti-tumor response can be generated against a self-protein in wild type FVB/N mice (Singh et al., 2005).

We continued the study of the anti-tumor efficacy of the five previously described *Listeria* based HER-2/neu vaccines (Figure 1).



EC1: AA 20-326
EC2: AA 303-501
EC3: AA 479-655

IC1: AA 690-1081
IC2: AA 1020-1260

Figure 1. Schematic of the overlapping extracellular and intracellular fragments of HER-2/neu cloned into an expression vector. The numbers represent amino acids.

Anti-tumor responses were initially studied through the analysis of the lysis of cells lines expressing fragments of HER-2/neu by vaccine stimulated $CD8^+$ T cells. This analysis led to the identification of regions of HER-2/neu that contain previously unidentified $CD8^+$ T cell epitopes for the FVB mouse (Table 1).

Listeria Construct	Neu Region Spanned	Killing of 3T3-neu Target Cells[a]	Neu Regions Containing an Epitope
Lm-LLO-EC1	30-326	**Neu-1**/Neu-2/*Neu-3*	20-148/291-326
Lm-LLO-EC2	303-501	*Neu-3*/**Neu-4**	303-426/401-501[b]
Lm-LLO-EC3	479-655	**Neu-4**/**Neu-5**	479-553/531-655
Lm-LLO-IC1	690-1081	*Neu-6*/Neu-7/*Neu-8*	690-797/952-1081
Lm-LLO-IC2	1020-1260	*Neu-8*/**Neu-9**	1020-1085/1063-1260

Table 1. Regions of HER-2/neu with potential FVB epitopes based on specific lysis from CTL analysis and the corresponding vaccine constructs. [a] **Strong killing: greater than 20% above background lysis**; *weak killing: 10-20% above background lysis*; no killing above background lysis. Background lysis varies between 0-12% for each experiment. [b]The epitope identified from Ercolini et al. (Ercolini et al., 2003) is from 420-429 and is partially contained in Neu-3 and fully in Neu-4.

Following the identification of regions of HER-2/neu with potential epitopes, we had overlapping 20mer peptides made to span all of the regions identified through the use of the cell lines containing fragments of HER-2/neu. These peptides have since been used to further narrow down the regions of HER-2/neu containing epitopes. CTL analyses of all 58 peptides were done using pools of 3-4 peptides at a time, which allowed us to narrow down these larger portions of HER-2/neu into separate regions spanning about 60 amino acids. The pools are now being split into individual 20mers to further narrow down regions containing $CD8^+$ T cell epitopes. This work has been summarized into a table showing the progression from using cell lines to narrow down target regions to the use of peptides to identify the 20mers containing epitopes (Table 2).

3T3 regions	Region with epitope	Peptide pools	Response to pool	Region with epitope
1-165	20-148	20-39/35-54/50-69	yes	20-69
		65-84/80-99/95-114	no	
		110-129/125-144/140-148	yes	110-148
148-291				
274-426	291-326	291-310	yes	291-310
274-426	303-426	291-310/366-385/381-400/396-415	no	
410-553	410-501	411-430/426-445/441-465	yes	411-465
		456-475/471-490/486-505	yes	456-501
410-553	479-553	471-490/486-505/501-520	no	
		516-535/531-550/546-565	yes	516-565
531-687	531-655	516-535/531-550/546-565	yes	516-565
		561-580/576-595/591-610	yes (small)	561-610
		606-625/621-640/636-655	yes (small)	606-655
665-820	690-797	690-709/705-724/720-739	no	
		735-754/765-784/780-797	yes	735-797
797-952				
929-1085	952-1081	967-987/982-1001/997-1016	no	
		1012-1031/1027-1046/1042-1061	yes	1012-1061
		1057-1076/1072-1091	no	
929-1085	1020-1085	1012-1031/1027-1046/1042-1061/1057-1076	yes	1012-1076
1063-1259	1063-1259	1072-1091/1087-1106/1102-1121	yes	1072-1121
		1117-1136/1132-1151/1147-1166	yes	1117-1166
		1162-1181/1177-1196/1192-1211	yes	1162-1211
		1207-1226/1222-1241/1238-1257	no	

Table 2. Summary of CTL analysis to date using cell lines and pools of 20mer peptides to narrow down regions of HER-2/neu potentially containing $CD8^+$ T cell epitopes.

In addition to this, one epitope has been fully mapped and we are in the process of studying it in comparison to the previously defined 'immunodominant' epitope (Ercolini et al., 2003). As can be seen in Table 2, there was a response to one 20mer, from 291-310, that was tested alone and not in a pool of peptides. Based on this result, this 20mer was divided into 9 and 10mers that were predicted by Rankpep to bind to MHC I molecules that are similar in structure to the "q" haplotype (Lee et al., 1988), and these were then tested to see if there was a CTL response to one of these smaller peptides (Figure 2). The FVB/N mouse is on the "q" background and not much is known about these MHC molecules, so at this point there are no programs that will directly predict the sequence of q binding peptides.

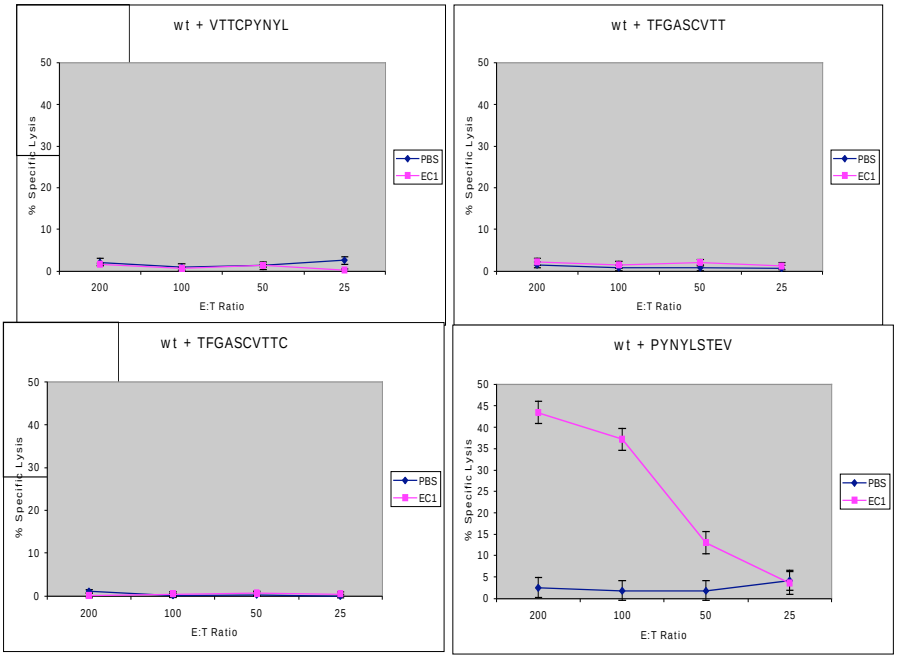


Figure 2. CTL analysis of 3T3-wt cells loaded with 9 and 10mer peptides corresponding to the 291-310 region of HER-2/neu. The peptide loaded onto the wild type 3T3 cells is noted above each of the graphs.

This analysis shows that a previously unidentified epitope for HER-2/neu that falls into the EC1 region of the *Listeria* based vaccines is defined by the amino acid sequence of PYNYLSTEV. The FVB/N mouse is on the H-2^q background and the previously determined "Ercolini" epitope has been determined to be restricted to the H-$2D^q$ class I MHC. In order to determine what the MHC restriction of this EC1 epitope is, we obtained cell lines that express only either H-$2D^q$ or H-$2L^q$ class I molecules to test in a CTL assay. As there is no cell line that only expresses H-$2K^q$, binding of the peptide to just this MHC class I molecule cannot directly be tested. We obtained an antibody that will specifically block H-$2K^q$ to determine if by blocking this MHC class I molecule lysis of target cells pulsed with this peptide is abrogated (Figure 3).

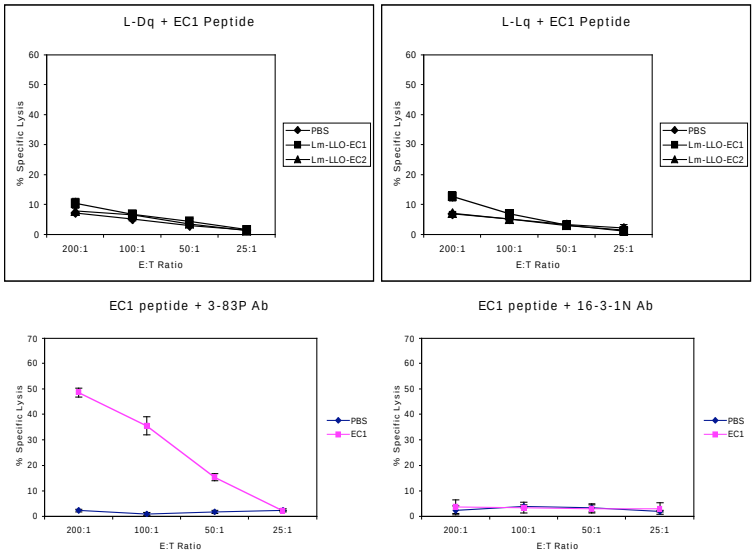


Figure 3. The MHC class I restriction of the EC1 epitope PYNYLSTEV is H-2K^q.

The top two panels show that there is no lysis of target L-L^q or L-D^q cells labeled with the EC1 peptide, which means that this epitope is not restricted to either H-2L^q or H-2D^q. The bottom two panels are of CTL analyses with 3T3-wt cells as targets and these cells express all three (L^q, D^q, and K^q) MHC class I molecules. 3-83P is an antibody that does not bind and block the binding groove of these class I molecules and most importantly does not bind to H-2K^q. 16-3-1N is an antibody that specifically binds into the binding groove of H-2K^q, and thus blocks binding of the peptide, but does not bind to either H-2L^q or H-2D^q. When the peptide is loaded on cells treated with 3-83P, there is still killing of the target cells by cells stimulated with the EC1 vaccine, but when the cells are treated with the 16-3-1N antibody, this binding is abrogated, as evidenced by the lack of killing of the target cells. This clearly shows that the MHC class I restriction of the PYNYLSTEV peptide is H-2K^q.

Through these CTLs, we have identified regions where there is clearly specific lysis of the target cells, implying that these regions contain unidentified CD8$^+$ T cell epitopes, and also regions where there is no lysis at all. We have previously theorized that these 'sub-dominant' epitopes are revealed by (1) breaking HER-2/neu into fragments; (2) fusing these fragments to listeriolysin O (LLO); (3) delivering the antigen through *Listeria monocytogenes*; or (4) a combination of these. In order to determine which of these is correct, we made and tested DNA vaccines to compare the full length HER-2/neu with a representative fragment, and also compare LLO-fused with non-fused antigens. These DNA vaccines were made using the pcDNA 3.1 parent vector and then tested in a tumor regression experiment with Lm-LLO-EC1 was a *Listeria* delivery control. The data for this experiment are extensive and are contained within the Journal of Immunology paper in the appendix to this report (Singh et al., 2005). This experiment shows that the vaccine delivered by *Listeria* is the best and the LLO-fused DNA vaccines are far superior to the non-fused vaccines. Thus, fusion to LLO and delivery by *Listeria*

monocytogenes both play a role in the revelation of 'sub-dominant' epitopes for HER-2/neu in the FVB mouse.

Much of the work done this year has focused on the FVB HER-2/neu transgenic mouse as a follow-up to the work discussed in my last annual report showing a significant slowing in the growth of implanted tumors after vaccination compared to the control groups. This experiment was first repeated to see if the mice in the HER-2/neu vaccine groups once again scratch away their tumors at around day 40 in what appears to be a delayed type hypersensitivity (DTH) response (Figure 4).

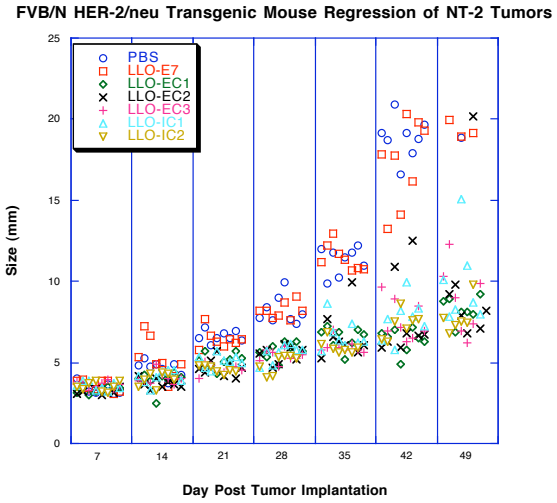


Figure 4. Tumor growth of implanted NT-2 tumors in FVB HER-2/neu transgenic mice. Mice were vaccinated with the noted vaccines on days 7, 14, 21, 28, and 35. Tumor growth was measured until the mice in the LLO-EC1, LLO-EC2, LLO-EC3, LLO-IC1, and LLO-IC2 groups scratched away their tumors.

This shows that the vaccines stimulate a response that is strong enough to overcome tolerance, as the growth of the implanted tumors is significantly slower in the Lm-LLO-HER-2/neu vaccine groups than those in the control *Listeria* vaccine group or the PBS control group. Due to the breaking of tolerance in a transplanted tumor model, we sought to test the efficacy of these vaccines in an autochthonous tumor model. 90% of female FVB/N HER-2/neu transgenic mice develop spontaneous mammary tumors between 4 and 9 months of age. In an autochthonous tumor test, the mice are treated prior to the development of tumors and observed for an extended period of time to see when they develop tumors and this is compared to the onset of tumor growth of control treated and untreated mice to look for differences in tumor growth. Age matched female mice were first vaccinated at 6 weeks of age and then every three weeks after that for a total of 5 vaccinations, resulting in treatments at 6, 9, 12, 15, and 18 weeks of age (Figure 5).

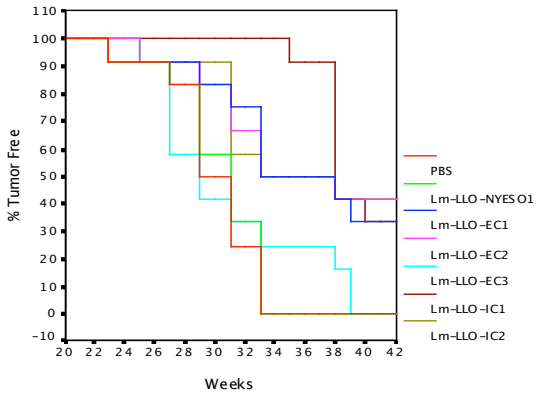


Figure 5. Autochthonous tumor protection in female FVB/N HER-2/neu transgenic mice. 12 mice per group were treated starting at 6 weeks of age and were followed until the onset of tumor growth in each mouse.

The delay in the onset of tumor growth in mice treated with Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-IC1, and Lm-LLO-IC2 was significantly different than either of the control groups (PBS and Lm-LLO-EC3). Lm-LLO-EC3 was not significantly different than either of the controls (Table 3).

	p-value
PBS vs NYESO	0.6742
PBS vs EC1	0.0036
PBS vs EC2	0.0062
PBS vs EC3	0.5685
PBS vs IC1	0
PBS vs IC2	0.0054

	p-value
NY vs EC1	0.0065
NY vs EC2	0.0112
NY vs EC3	0.6962
NY vs IC1	0
NY vs IC2	0.0108

	p-value
IC1 vs EC1	0.3859
IC1 vs EC2	0.5535
IC1 vs EC3	0.0006
IC1 vs IC2	0.5875

Table 3. Kaplan-Meier Log Rank Test. p-values showing which groups are significantly different in the autochthonous tumor protection are shown.

These vaccines are clearly capable of breaking tolerance in FVB/N HER-2/neu transgenic mice based on the significant delay seen in the onset of spontaneous tumor growth (Figure 5) and also in the slowing of tumor growth with transplanted tumors (Figure 4). The apparent discrepancy between these tumor results is a current area of study. When tumors are implanted in the transgenic mice, treatment with each of the vaccines leads to the same result, a slowing in the growth of the tumors and the eventual scratching away of the tumors in all of the Lm-LLO-HER-2/neu vaccinated groups around day 40 (Figure 4). In addition, the tumor sizes in all of the mice in the Lm-LLO-HER-2/neu vaccine groups are clustered together with no difference between any of the treatment groups. Conversely, clear differences between the efficacy of the vaccines can be seen through the autochthonous tumor protection. Lm-LLO-EC3 is not efficacious in this type of experiment, while Lm-LLO-IC1 is definitively the best vaccine in this model (Figure 5).

There are several factors that could explain the differences in the efficacy of the vaccines in these experiments. In one case tumors are implanted and mice are treated after the

growth of palpable solid tumors (about 4-5 mm in size), while in the other, mice are treated prior to the development of natural tumors. This means that in the first case (Figure 4) the efficacy of the vaccines as a therapeutic is being tested, while in the other experiment (Figure 5), the efficacy of the vaccines as a prophylactic is being determined. Another difference between these two tests of the vaccines is that the spontaneous tumors develop in the mammary glands of the mice, whereas when tumors are implanted, they are placed subcutaneously on the flank of the mice. The differences in the efficacy of the vaccines in these experiments comes down to potential differences in the stromal environment of the tumors and also the timing of the vaccine. In the autochthonous tumor experiment, although mice are first vaccinated at 6 weeks of age, some of the mice will already be in the process of developing mammary tumors through hyperplasia of the mammary glands by this time.

For the coming year, several experiments have been planned and questions of interest have been identified. We are in the process of trying to determine the factors that contribute to the increased efficacy of Lm-LLO-IC1 vs Lm-LLO-EC3 in the autochthonous tumor protection by conducting a tumor regression experiment where mice are given tumors either subcutaneously or in the mammary gland and then treated with the vaccines to determine if stromal elements are involved in the efficacy of the Lm-LLO-HER-2/neu vaccines. Another question of interest is to determine if mutations in the HER-2/neu protein contribute to the onset of spontaneous tumor growth in the Lm-LLO-HER-2/neu vs control vaccinated mice. In order to do this, we are currently sequencing tumor samples to see if mutations can be seen.

III. Key Research Accomplishments

- CTL analysis of each vaccine with pools of peptides corresponding to regions of HER-2/neu previously identified to contain a potential epitope through the use of cell lines.
- Identification of a novel $CD8^+$ T cell epitope for rat HER-2/neu in the FVB/N mouse, PYNYSTLEV.
- Determination of the MHC class I restriction of this novel epitope ($H\text{-}2K^q$).
- Repeat regression study in HER-2/neu transgenic FVB mice with implanted NT-2 tumors showing a slower rate of tumor growth in Lm-LLO-HER-2/neu vaccinated mice vs control treated groups.
- Autochthonous tumor protection in FVB HER-2/neu transgenic mice showing a significant delay in the onset of tumor growth for the Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-IC1, and Lm-LLO-IC2 vaccines.

IV. Reportable Outcomes

- Publication of a research article in the Journal of Immunology.

- Presentation of a poster based on this data at the Keystone Tumor Immunology meeting in March 2005.
- Platform presentation given based in this data at the Department of Defense ERA of Hope meeting in June 2005.
- Presentation of a poster based on this data at the Department of Defense ERA of Hope meeting in June 2005.
- Presentation of a poster based on this data at the Cancer Research Institute meeting in October 2005.
- Patent application ongoing.

V. Conclusions

Listeria monocytogenes based vaccines for HER-2/neu are capable of breaking tolerance and leading to a slowing in the growth of transplanted tumors in FVB/N HER-2/neu transgenic mice. Treatment with each of these vaccines results in the slowing of tumor growth compared to control groups and then the mice in the HER-2/neu vaccine groups scratch their tumors away around day 40. Tolerance in very clearly broken in this transgenic model in the case of autochthonous tumors, with a significant delay in the onset of tumor growth with mice vaccinated with Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-IC1, or Lm-LLO-IC2. In this type of protection experiment, Lm-LLO-EC3 is ineffective and does not lead to a delay in the onset of the growth of spontaneous tumors. The factors underlying the differences in the anti-tumor efficacy of these vaccines in a regression of transplanted tumors vs a protection of spontaneous tumors are currently unknown. We are in the process of determined if tumor stromal elements are involved in the anti-tumor response of these vaccines. The eventual growth of autochthonous tumors and the factors that lead to this are another area of current study.

Continuing and following up on the previously discussed cytotoxic T lymphocyte analyses has led to the identification of one novel $CD8^+$ T cell epitope for rat HER-2/neu in the FVB/N mouse, to date. This work is also currently ongoing. We plan to identify several epitopes spanning distinct regions of HER-2/neu. Identification of several epitopes could lead to the next generation of *Listeria monocytogenes* based vaccines for HER-2/neu containing specific regions of HER-2/neu corresponding to these epitopes. In this way the vaccines would be able to elicit immune responses specifically targeted towards epitopes spread throughout the entire HER-2/neu protein with a single vaccine.

In the past year, testing of these vaccines in transgenic mice that are tolerant to rat HER-2/neu has shown the ability of these vaccines to overcome tolerance in these mice. This is an important step in the development of therapies that may be adapted for humans as any therapy used in humans must be capable of generating a strong enough immune response to break tolerance in order to significantly impact on tumor growth in any way.

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VII. Appendix

The Journal of Immunology

Fusion to Listeriolysin O and Delivery by *Listeria monocytogenes* Enhances the Immunogenicity of HER-2/neu and Reveals Subdominant Epitopes in the FVB/N Mouse[1]

Reshma Singh,* Mary E. Dominiecki,[2]* Elizabeth M. Jaffee,† and Yvonne Paterson[3]*‡

Five overlapping fragments of rat HER-2/neu have been expressed in recombinant *Listeria monocytogenes*. Each fragment of HER-2/neu is secreted as a fusion protein with a truncated, nonhemolytic form of listeriolysin O (LLO). Lm-LLO-EC1, Lm-LLO-EC2, and Lm-LLO-EC3 overlap the extracellular domain of HER-2/neu, whereas Lm-LLO-IC1 and Lm-LLO-IC2 span the intracellular domain. All five strains controlled the growth of established NT-2 tumors, a rat HER-2/neu-expressing tumor line derived from a spontaneously arising mammary tumor in a FVB/N HER-2/neu-transgenic mouse. The antitumor effect of each of these vaccine constructs was abrogated by the in vivo depletion of $CD8^+$ T cells, although only one known epitope has been defined previously and is present in Lm-LLO-EC2. Anti-HER-2/neu CTL responses were generated by each of the rLm vaccine constructs. With the use of a panel of 3T3 cell lines expressing overlapping fragments of HER-2/neu, regions of HER-2/neu with potential $CD8^+$ T cell epitopes have been defined. DNA vaccines expressing either a fragment or full-length HER-2/neu were constructed in LLO-fused and non-LLO-fused forms. CTL analysis of the DNA vaccines revealed a broadening in the regions of HER-2/neu recognizable as targets when the target Ag is fused to LLO. These studies show the efficacy of *L. monocytogenes*-based HER-2/neu vaccines in a murine model of breast cancer and also that the immunogenicity of self-Ags can be increased by fusion to LLO and delivery by *L. monocytogenes* revealing subdominant epitopes. *The Journal of Immunology*, 2005, 175: 3663–3673.

A member of the epidermal growth factor receptor family of tyrosine kinases, HER-2/neu is a 185-kDa glycoprotein. It consists of an extracellular domain, a transmembrane domain, and an intracellular domain, which is known to be involved in cellular signaling (1–4). It is overexpressed in 25–40% of all breast cancers and is also overexpressed in many cancers of the ovaries, lung, pancreas, and gastrointestinal tract (5–7). The overexpression of HER-2/neu is associated with uncontrolled cell growth and signaling, both of which contribute to the development of tumors (2, 8). Patients with cancers that overexpress HER-2/neu exhibit tolerance even with detectable humoral (9), $CD8^+$ T cell (10), and $CD4^+$ T cell (11) responses directed against HER-2/neu.

Listeria monocytogenes is an intracellular pathogen that primarily infects APCs and has adapted for life in the cytoplasm of these cells (12, 13). Host cells, such as macrophages, actively phagocytose *L. monocytogenes*, and the majority of the bacteria are degraded in the phagolysosome (14). Some of the bacteria escape into the host cytosol by perforating the phagosomal membrane through the action of a hemolysin, listeriolysin O (LLO)[4] (13, 15). Once in the cytosol, *L. monocytogenes* can polymerize the host actin and pass directly from cell to cell further evading the host immune system and resulting in a negligible Ab response to *L. monocytogenes* (13).

L. monocytogenes is an attractive vaccine vector because proteins produced by this bacterium can be presented as short peptides via both the MHC class I and class II pathways generating both $CD8^+$ and $CD4^+$ T cell responses to these Ags (16). Direct killing of tumor cells occurs through the function of $CD8^+$ T cells, but this killing may be enhanced through the activation of $CD4^+$ T cells. MHC class II molecules present peptides derived from listerial proteins in the phagolysosome to activate $CD4^+$ T cells (16). Conversely, proteins that are produced by *L. monocytogenes* in the host cytosol are presented to $CD8^+$ T cells by MHC class I molecules (17, 18). Taking advantage of the ability of *L. monocytogenes* to target Ags to both of these pathways should lead to a strong cellular immune response against the Ags it produces. Our lab has previously shown this in response to the human papillomavirus (HPV) E7 protein and the influenza nucleoprotein (NP) (17, 19–21). Both of these systems used recombinant *L. monocytogenes* containing a plasmid with a truncated, nonhemolytic, LLO fused to the N terminus of either E7 (Lm-LLO-E7) or NP (Lm-LLO-NP) (17, 19). In both these cases, complete tumor regression can be seen upon vaccination of mice-bearing tumors with the target Ag (19, 20). In particular, Lm-LLO-E7 induces the complete regression of >75% of established E7-expressing murine tumors. This vaccine is also capable of generating an E7-specific $CD8^+$ T cell response that can be used to effectively kill target tumor cells in a CTL assay, and the antitumor response is abrogated in vivo upon depletion of $CD8^+$ T cells (19).

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[4] Abbreviations used in this paper: LLO, listeriolysin O; HPV, human papillomavirus; NP, nucleoprotein; PEST, proline, glutamic acid, serine, and threonine.

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Based on these results, we have adapted the Lm-LLO-Ag system for the self-Ag HER-2/neu. Several other vaccines using the HER-2/neu tumor Ag in the mouse have been studied previously. In wild-type FVB/N mice, whole cell vaccination has been effective in preventing tumor growth upon tumor challenge but is not effective as a treatment for established tumors (22). Recombinant vaccinia virus transduced with HER-2/neu has also been shown to be fairly effective in a tumor prevention model (23). HSP-110-HER-2/neu chaperone complex vaccines containing the intracellular domain of HER-2/neu have also been described previously (24). It has been shown previously that depletion of either $CD4^+$ or $CD8^+$ T cells in tumor-bearing mice results in decreased tumor regression vs undepleted mice (25). Because of the ability of *L. monocytogenes* to generate both an Ag-specific $CD4^+$ or $CD8^+$ T cell response, it appears to be a promising vaccine vector for HER-2/neu. In addition, the use of a bacterium such as *L. monocytogenes* to deliver fragments of HER-2/neu fused to LLO may make self-Ags, such as HER-2/neu, immunogenic enough to induce an anti-HER-2/neu tumor response in mice with HER-2/neu-positive tumors.

In this study, we describe a series of *L. monocytogenes*-based vaccines for HER-2/neu. Each of these vaccines is capable of significantly impacting on tumor growth and is able to induce an anti-HER-2/neu CTL response, despite the fact that there is only one known $CD8^+$ T cell epitope, which falls into a region covered by only one vaccine (Lm-LLO-EC2) (26). These results suggest that there are subdominant HER-2/neu epitopes that are revealed through this vaccination strategy that are immunogenic enough to induce $CD8^+$ T cells that can control tumor growth. This study further attempts to determine whether these subdominant epitopes are revealed through 1) the use of *L. monocytogenes* as a vaccine vector, 2) fusion of the Ag to LLO, 3) splitting HER-2/neu into fragments, or 4) a combination of these possibilities.

Materials and Methods

L. monocytogenes *and vaccine construction*

The *L. monocytogenes* strains used for these studies are Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2, all of which contain a fragment of rat HER-2/*neu* fused to the listerial *hly* gene in an episomal expression system that has been described previously by our lab (19, 21). Fragments of HER-2/*neu* have been cloned into the plasmid pGG-55, and the amino acids in each fragment are 20–326 in Lm-LLO-EC1, 303–501 in Lm-LLO-EC2, 479–655 in Lm-LLO-EC3, 690-1081 in Lm-LLO-IC1, and 1020–1260 in Lm-LLO-IC2 (see Fig. 1*A*). Each fragment was amplified by PCR from the pNINA plasmid, which contains the full-length rat HER-2/*neu* gene using the following primers: EC1 (bp 74–994) 5′-CACGCGGATGAAATCGATAAGCTCGAGCCCCCCGGAATCGCGG GCAC (*Xho*I site underlined) and 3′-CCGGACTAGTGACCTCTTGGTTA TTCGGGGGACACACC (*Spe*I site underlined); EC2 (bp 923-1519) 5′-CC GGGTCGACTGCCCCTACAACTACCTGTCTACG (*Sal*I site underlined) and 3′-CCGGACTAGT*TTACTTGTCATCGTCGTCCTTGTAGTC*CCCCACT GTGGAGCAGGGCCTG (*Spe*I site underlined, FLAG tag sequence in italics); EC3 (bp 1451–1981) 5′-CCGGGTCGACTGCTTTGTACACACTGTA CCTTGG (*Sal*I site underlined) and 3′-CCGGACTAGT*TTACTTGTCA TCGTCGTCCTTGTAGTC*CGGGCTGGCTCTCTGCTCTGC (*Spe*I site underlined, FLAG tag in italics); IC1 (bp 2084–3259) 5′-CCGG CTCGAGTATACGATGCGTAGGCTGCTGCAGG (*Xho*I site underlined) and 3′-CCGGACTAGTAGCCAGTGGAGATCTGGGGGGCCC (*Spe*I site underlined); and IC2 (bp 3073–3796) 5′-CCGGCTCGAGGGTGACCTGG TAGACGCTGAAG (*Xho*I site underlined) and 3′-CCGGACTAGTTACAG GTACATCCAGGCCTAGG (*Spe*I site underlined). Each of these fragments was amplified by PCR and cloned into the pCR 2.1 expression system (Invitrogen Life Technologies). Each fragment was then excised from the pCR2.1 plasmid with the delineated enzymes. For all the constructs, the *E7* gene was excised from the pGG-55 plasmid (19) with *Xho*I and *Spe*I. Each HER-2/neu fragment was then ligated into the E7 site (ends cut with *Sal*I are compatible with *Xho*I cuts). The XFL-7 strain (19) of *L. monocytogenes* was then electroporated with each plasmid. Bacteria were grown in brain heart infusion medium (BD) with 50 μg/ml chloramphenicol and then frozen in 1-ml aliquots at −80°C. The LD_{50} of each vaccine was determined as previously described by vaccinating mice with various doses of each vaccine construct (19). The LD_{50} of each vaccine construct is as follows: 1×10^8 CFU for Lm-LLO-EC1, 5×10^8 CFU for Lm-LLO-EC2, 1×10^9 CFU for Lm-LLO-EC3, 1×10^8 CFU for Lm-LLO-IC1, and 1×10^8 CFU for Lm-LLO-IC2.

Lm-LLO-E7 as used as a control for non-Ag-specific effects throughout this study. This strain is isogenic with the Lm-LLO-HER-2/neu strains, except for the Ag expressed and has been described previously (19).

The DNA vaccines were all inserted into the pcDNA 3.1 plasmid (Invitrogen Life Technologies). Full-length HER-2/*neu* and the *EC1* fragment were amplified by PCR for both the LLO-fused and non-LLO-fused versions of the DNA vaccine. The primers used were as follows: full-length HER-2/*neu* no fusion 5′-CCGGGCTAGCATGGTCATCATGGAGCTG GCCGG (*Nhe*I site underlined) and 3′-CCGGGATATC*TTACTTGT CATCGTCGTCCTTGTAGTC*TCATACAGGTACATCCAGGCC (*Eco*RV site underlined, FLAG tag in italics, stop codon in bold); full-length HER-2/*neu* fused to LLO 5′-CCGGGTCGACATGGTCATCATGGAGCTG GCCGG (*Sal*I site underlined) and the same 3′ primer as for the full-length HER-2/*neu* with no fusion; *EC1* no LLO fusion 5′ primer same as full-length HER-2/*neu* no fusion 5′ primer and 3′-CCGGGATATC*TTACTTGTCATCG TCGTCCTTGTAGTC*TCAGACCTCTTGGTTATTCGGGGG (*Eco*RV site underlined, FLAG tag in italics, stop codon in bold); *EC1* with LLO fusion 5′ primer same as the 5′ primer used for the full-length HER-2/neu with LLO fusion and 3′ primer same as the 3′ primer for *EC1* no LLO fusion. All the fragments were cloned into the multicloning site of pcDNA3.1 downstream from the CMV promoter either containing LLO or not. *Escherichia coli* were transformed with each plasmid and the bacteria were grown in Luria-Bertani medium (BD) with 50 μg/ml ampicillin.

Western blotting

The HER-2/neu *L. monocytogenes* vaccines were grown overnight in Luria-Bertani medium with 50 μg/ml chloramphenicol at 37°C. Supernatants were TCA precipitated and resuspended in 1× LDS sample buffer (Invitrogen Life Technologies). Fifteen microliters of each sample were loaded on a 4–12% Bis-Tris SDS-PAGE gel (Invitrogen Life Technologies). The gel was then transferred to a Immobilon-P polyvinylidene fluoride membrane (Millipore) and blotted with a polyclonal rabbit serum raised to the first 30 residues of the LLO protein (anti-proline, glutamic acid, serine, and threonine (PEST)). The secondary Ab was an HRP-conjugated anti-rabbit Ab (Amersham Biosciences).

Mice

Six- to 8-wk-old female FVB/N mice were purchased from Charles River Laboratories.

Cell lines

The FVB/N syngeneic NT-2 tumor cell line was developed from a spontaneously occurring mammary tumor in a FVB/N HER-2/neu-transgenic mouse (27). NT-2 tumor cells constitutively express low levels of rat HER-2/neu and are tumorigenic in wild-type syngeneic mice. NT-2 cells were grown in RPMI 1640 medium supplemented with 20% FCS, 10.2 mM HEPES, 2 mM L-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 U/ml penicillin G, 50 μg/ml streptomycin, 20 μg/ml insulin, and 2 μg/ml gentamicin at 37°C with 5% CO_2. NIH 3T3 cells are a mouse fibroblast line and the 3T3-neu lines were made from these wild-type cells as described previously (26). Briefly, wild-type 3T3 cells were transduced with overlapping fragments of the rat HER-2/neu gene, creating nine 3T3 HER-2/neu fragment lines, and one 3T3 line expressing the full-length rat HER-2/neu. The 3T3 HER-2/neu fragment lines encompass the following overlapping amino acid regions: 3T3-neu-1 spans 1–165; 3T3-neu-2 spans 148–291; 3T3-neu-3 spans 274–426; 3T3-neu-4 spans 410–553; 3T3-neu-5 spans 531–687; 3T3-neu-6 spans 665–820; 3T3-neu-7 spans 797–952; 3T3-neu-8 spans 929-1085; and 3T3-neu-9 spans 1063–1260. The NIH 3T3 and all the derived cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 U/ml penicillin G, and 50 μg/ml streptomycin. Culture medium for the 3T3-neu cell lines was supplemented with 1 mg/ml G418. Cells were grown at 37°C with 5% CO_2.

Tumor regression

Six- to 8-wk-old FVB/N mice were injected s.c. on the right flank with 2×10^6 NT-2 tumor cells in 200 μl of PBS. One week posttumor inoculation, the tumors reached a palpable size at 4–5 mm. Each treatment group consisted of eight mice, which were vaccinated with 0.1 LD_{50} i.p. Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, Lm-LLO-E7, or PBS on days 7, 14, and 21 after tumor inoculation.

Measurement of tumor growth

Tumors were measured every 2 days with calipers spanning the shortest and longest surface diameters. Plots show the mean of these two measurements as the tumor size in millimeters vs time. Mice were sacrificed when mean tumor diameter reached 20 mm, and tumor measurements for each time point are shown only for the surviving mice.

$CD8^+$ cell depletion

$CD8^+$ T cells were depleted in NT-2 tumor-bearing mice with 0.5 mg of 2.43 (21) on days 6, 7, 8, 11, 14, 17, 20, and 23 postlumor injection. The 2.43 (anti-$CD8^+$ T cell) Ab was affinity purified from ascites on a protein G-Sepharose column (Amersham Biosciences). This Ab has been shown by many investigators to specifically deplete only $CD8^+$ T cells. In contrast, a similarly purified, isotype-matched rat Ab, GL117.41, which produces an anti-*E. coli* β-galactosidase, had no effect on tumor growth in similar transplantable tumor models (19, 28). $CD8^+$ T cell populations were reduced by >95% as measured by flow cytometric analysis on day 24 (data not shown). Mice were vaccinated and tumors measured as described above.

Flow cytometric analysis

FVB/N mice, 6–8 wk old, were immunized with either PBS, 0.1 LD_{50} Lm-LLO-EC2, or 0.1 LD_{50} Lm-LLO-E7 and then boosted 21 days later. Three-color flow cytometry for CD8 (53-6.7, FITC conjugated), CD62 ligand (Mel-14, APC conjugated) (BD Biosciences), and HER-2/neu $H\text{-}2^q$ tetramer (PE conjugated) was performed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences). The tetramer was loaded with a $H\text{-}2^q$-specific PDSLRDLSVF peptide. Tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility of Emory University (Atlanta, GA), under the direction of Dr. J. Altman, and the National Institutes of Health AIDS Research and Reference Reagent Program. Splenocytes were harvested 5 days after the boost and were stained at room temperature with the tetramer for 1 h at a 1/200 dilution. Cells were then stained on ice with anti-CD8 and anti-CD62L Abs for 30 min. The splenocytes were then analyzed as described above comparing $CD8^+$,$CD62L^{low}$,tetramer$^+$ cells generated by either PBS, Lm-LLO-EC2, or Lm-LLO-E7 vaccination. Analysis was done using FlowJo software (Tree Star).

^{51}Cr release assay

Six- to 8-wk-old FVB/N mice were immunized i.p with 0.1 LD_{50} Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, Lm-LLO-E7, or PBS or with 50 μg i.m. of pcDNA neu, pcDNA LLO-neu, pcDNA EC1, or pcDNA LLO-EC1. Mice were then sacrificed 9 days after immunization, and spleens were harvested. Splenocytes were then cultured with irradiated NT-2 (20,000 rad) tumor cells at a 100:1 ratio of splenocytes to tumor cells with 20 U/ml IL-2 (Roche). Following 4 days of culture, the splenocytes were used as effector cells in a standard ^{51}Cr release assay. Briefly, target cells (3T3-wt, 3T3-neu, 3T3-neu-1, 3T3-neu-2, 3T3-neu-3, 3T3-neu-4, 3T3-neu-5, 3T3-neu-6, 3T3-neu-7, 3T3-neu-8, and 3T3-neu-9 lines) were labeled with chromium and were then cultured with splenocytes at the E:T ratios of 200:1, 100:1, 50:1, and 25:1 in triplicate for 4 h. In addition, 3T3-wt cells were pulsed for a minimum of 1 h before labeling with ^{51}Cr with 1 μM of the $H\text{-}2^q$-specific PDSLRDLSVF peptide. Following the incubation, 100 μl of supernatant were assayed for ^{51}Cr release. The percent-specific lysis was determined as ((experimental counts per minute − spontaneous counts per minute)/(total counts per minute − spontaneous counts per minute)) × 100.

DNA vaccination

Female FVB/N mice that were 6–8 wk old were given 7×10^5 NT-2 tumor cells s.c. on the right flank. Mice were then vaccinated i.m. with either 50 μg of GM-CSF, 50 μg of pcDNA neu + GM-CSF, 50 μg of pcDNA LLO-neu + GM-CSF, 50 μg of pcDNA EC1 + GM-CSF, or 50 μg of pcDNA LLO-EC1 + GM-CSF or i.p. with PBS or 0.1 LD_{50} Lm-LLO-EC1. The vaccines were given on days 3, 10, and 18 and tumors were measured every 3 days. Mice were sacrificed when their tumors reached 20 mm in size.

Statistics

The Student *t* test was used for statistical analyses, and significant differences ($p < 0.05$) are noted.

Results

Generation of L. monocytogenes *strains that secrete fragments of HER-2/neu*

Five recombinant *L. monocytogenes* strains that express and secrete overlapping fragments of the rat HER-2/neu gene have been designed and constructed (Fig. 1*A*). In the design of these vaccine constructs, the signal sequence and transmembrane domains of HER-2/neu have been deleted due to the hydrophobicity of these regions and the inability of *L. monocytogenes* to secrete extremely hydrophobic domains. All the fragments have been cloned into the pGG-55 expression system described previously (19). The Lm-LLO-HER-2/neu constructs have been designed based on the effectiveness of an artificial tumor Ag, NP, and the HPV tumor Ag, E7, in this system (19–21). The secretion of each of the HER-2/neu fragment vaccines has been confirmed by Western blot of secreted Listerial proteins (Fig. 1*B*). Secretion of the fusion protein LLO-E7 by Lm-LLO-E7 as a control is shown at 67 kDa. An 83-kDa recombinant protein is secreted by Lm-LLO-EC1, 70 kDa by Lm-LLO-EC2, 68 kDa by Lm-LLO-EC3, 92.5 kDa by Lm-LLO-IC1, and 74 kDa by Lm-LLO-IC2. Each of these strains is highly attenuated as compared with the wild-type 10403S, but the virulence of each strain is comparable to Lm-LLO-E7, which is $\sim 1 \times 10^9$ CFU.

Vaccination with each Lm-based HER-2/neu construct leads to stasis in tumor growth

Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2 were each compared with PBS controls and Lm-LLO-E7-vaccinated mice for their ability to impact on the growth

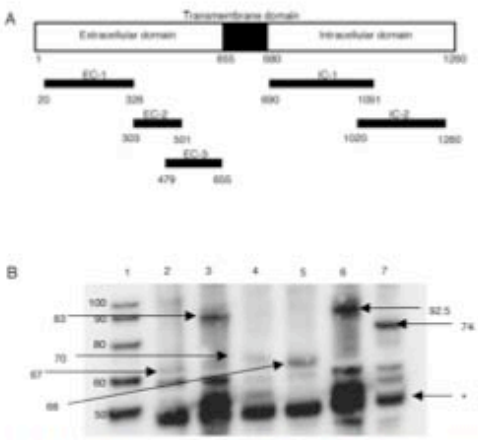

FIGURE 1. HER-2/neu has been broken into fragments, and recombinant *L. monocytogenes* is capable of secreting each of these vaccines as an LLO-fusion protein. *A*, Rat HER-2/neu was broken into fragments for the construction of a series of Lm-LLO-HER-2/neu vaccines as described under *Materials and Methods*. *B*, Following the construction of each of these vaccines, the secretion of the fusion peptides was confirmed by Western blot analysis. Marker (*lane 1*), Lm-LLO-E7 (*lane 2*), Lm-LLO-EC1 (*lane 3*), Lm-LLO-EC2 (*lane 4*), Lm-LLO-EC3 (*lane 5*), Lm-LLO-IC1 (*lane 6*), and Lm-LLO-IC2 (*lane 7*). * denotes the band for endogenous LLO secreted by all *L. monocytogenes*. The *Listeria* vaccines were grown overnight at 37°C in Luria-Bertani broth. Supernatants were TCA precipitated, and 15 μl of each sample were analyzed by Western blot analysis. The blot was probed with an anti-PEST rabbit serum, followed by HRP-conjugated anti-rabbit secondary Ab. The blot was developed using ECL detection reagents.

of the rat HER-2/neu-expressing tumor line, NT-2. FVB/N mice were given s.c. NT-2 tumor injections on the right flank that produced palpable tumors of 4–5 mm 7 days later. Mice were given weekly i.p. injections of 0.1 LD_{50} Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, or the controls Lm-LLO-E7 or PBS. Neither the PBS nor Lm-LLO-E7 injections had any impact on tumor growth, but within a week of the first vaccination with either Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, or Lm-LLO-IC2, NT-2 tumor growth became static and remained so until the experiment was terminated at 90 days (Fig. 2, *A* and *B*). This cessation in tumor growth remained well after the last HER-2/neu vaccination on day 21, and a subsequent regression of tumors in some of the mice was observed. Three of eight of the mice with Lm-LLO-EC2 and Lm-LLO-EC3 vaccinations were completely cured of their tumors, while one of eight mice vaccinated with Lm-LLO-EC1 or Lm-LLO-IC1 showed complete tumor regression. None of the tumors in the Lm-LLO-IC2 vaccinated group underwent a complete regression, but the tumor growth of the mice in this group remained static and in some cases the tumor size decreased.

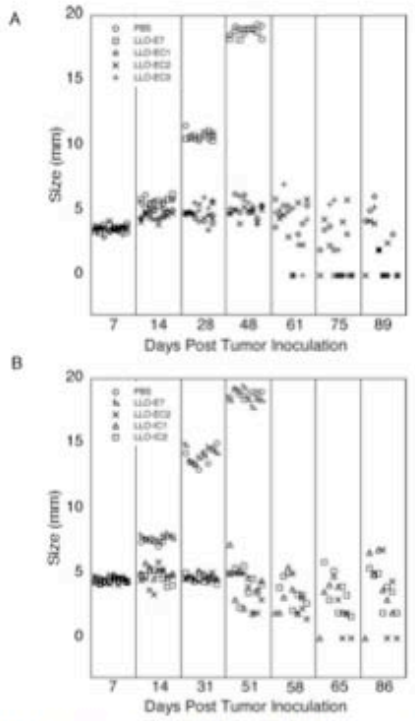

FIGURE 2. Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2 induce a halt in tumor growth of established NT-2 tumors. FVB/N mice (eight per group) were given 2×10^6 NT-2 cells by s.c. injection on the right flank. On days 7, 14, and 21 after tumor challenge, mice were treated with 0.1 LD_{50} Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, Lm-LLO-IC2, and Lm-LLO-E7 or 200 μl of PBS. Each data point represents the average tumor diameter of one mouse at a given time point as measured with calipers. Mice were sacrificed when the average tumor diameter reached 2.0 cm, and the tumor measurements for each time point are only shown for the surviving mice. Representative figures of two experiments are shown. Lm-LLO-EC1, Lm-LLO-EC2, and Lm-LLO-EC3 (*A*); Lm-LLO-IC1 and Lm-LLO-IC2 (*B*).

$CD8^+$ T cells are necessary for Lm-LLO-HER-2/neu vaccine effectiveness

For both Lm-LLO-E7 and Lm-LLO-NP, $CD8^+$ T cells are absolutely necessary for vaccine effectiveness (19). FVB/N mice were depleted of $CD8^+$ T cells after establishment of NT-2 tumors. Upon depletion of $CD8^+$ T cells with the anti-CD8 Ab 2.43, each of the Lm-LLO-HER-2/neu vaccines lost all effectiveness (Fig. 3, *A* and *B*). Tumor growth in mice that were not depleted of $CD8^+$ T cells could be controlled with vaccination of the mice with Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2 on days 7, 14, and 21. Growth of tumor in PBS mock-vaccinated mice from a similar experiment is shown in Fig. 6*C* for comparative purposes. The growth of tumor in PBS vaccinated mice is similar but slightly slower than the growth of tumors in the Lm-LLO-HER-2/neu-vaccinated mice that were depleted of $CD8^+$ T cells (Fig. 3*C*). This suggests that endogenous $CD8^+$ T cells induced by the tumor may be slowing tumor growth in unvaccinated mice as we have previously observed in other tumor models (21).

Lm-LLO-EC2 vaccination induces an increase in HER-2/neu-specific $CD8^+$ T cells

The $CD8^+$ T cell depletion tumor regression experiment shown in Fig. 3 clearly shows that $CD8^+$ T cells have an essential role in the control of NT-2 tumor growth. Measurement of the HER-2/neu-specific $CD8^+$ T cells activated through vaccination with the rLm constructs can be performed by tetramer analysis. This analysis is only possible for the Lm-LLO-EC2 construct because the only $CD8^+$ T cell epitope known for the FVB/N mouse is from aa 420–429, which is contained within the EC2 fragment (26). Mice were vaccinated with 0.1 LD_{50} Lm-LLO-EC2 or Lm-LLO-E7 or injected with PBS and then boosted 21 days later. Splenocytes were then stained with the $H\text{-}2^q$ tetramer loaded with the PD-SLRDLVF peptide, and the activated $CD8^+$ T cells were analyzed to determine the percentages that were tetramer positive (Fig. 4). A 5-fold increase in tetramer-positive cells in the spleens was observed in non-tumor-bearing FVB/N mice after vaccination with Lm-LLO-EC2 vs injection with either Lm-LLO-E7 or PBS.

CTL analysis shows the induction of $CD8^+$ T cells against subdominant epitopes

Because each Lm-LLO-HER-2/neu vaccine is capable of impacting on tumor growth, yet only one FVB/N $CD8^+$ T cell epitope has been described, CTL assays were performed using 3T3 cell lines expressing HER-2/neu fragments to determine which regions of HER-2/neu contain a potential epitope. Mice were immunized with one of the Lm-LLO-HER-2/neu vaccines, and the splenocytes were used as effector cells in a standard ^{51}Cr release assay. Initially each vaccine was tested with wild-type 3T3 cells as targets for a negative control and 3T3 cells transduced with full-length rat HER-2/neu (26) as a positive control. Each vaccine is capable of inducing an anti-HER-2/neu CTL response as evidenced by the specific lysis of 3T3-neu cells vs wild-type 3T3 cells (Fig. 5, *A* and *B*). Splenocytes from PBS-vaccinated mice produced little lysis regardless of the E:T ratio, while >20% lysis of 3T3-neu cells was produced by each of the Lm-LLO-HER-2/neu vaccine constructs at E:T ratios of 200:1 or 100:1. Splenocytes from PBS-vaccinated mice were used as a control for all subsequent CTL assays because no difference in lysis of 3T3-wt and 3T3-neu cells was seen using either PBS or Lm-LLO-E7-primed CTLs (Fig. 5, *C* and *D*). Both PBS and Lm-LLO-E7 vaccinations induced a very low level of

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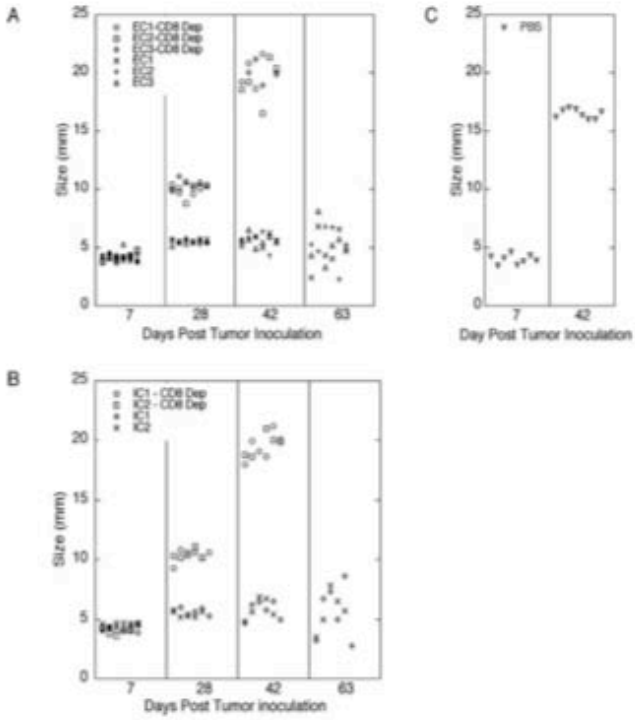

FIGURE 3. $CD8^+$ T cells are necessary for Lm-LLO-HER-2/neu-induced tumor stasis. FVB/N mice (six mice per group) were given 2×10^5 NT-2 cells by s.c. injection on the right flank. Mice were treated with 0.1 LD_{50} Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, or Lm-LLO-IC2 on days 7, 14, and 21 after tumor challenge. Mice were treated with 0.5 mg of 2.43 (anti-CD8 Ab) on days 6, 7, 8, 11, 14, 17, 20, and 23 following tumor challenge. Mice were sacrificed when tumor diameter reached ~2.0 cm, and the tumor measurements at each time point are shown only for the surviving mice. *A*, Lm-LLO-EC1, Lm-LLO-EC2, and Lm-LLO-EC3 both depleted and undepleted for $CD8^+$ T cells. *B*, Lm-LLO-IC1 and Lm-LLO-IC2 both depleted and undepleted for $CD8^+$ T cells. *C*, PBS-vaccinated mice that are not depleted of $CD8^+$ T cells.

nonspecific lysis that was virtually equal. The levels of lysis seen from the Lm-LLO-HER-2/neu vaccinations with any of the HER-2/neu-expressing cell lines is similar to the lysis observed by pulsing 3T3-wt target cells with the known FVB/N $CD8^+$ T cell epitope peptide at high E:T ratios (Fig. 5*E*). However, the lysis observed when not targeting a specific peptide titrates out by 50:1, whereas with a known peptide, a significant level of lysis can still be seen at an E:T ratio of 25:1 (Fig. 5*E*). This illustrates the fact that although these assays can be used as a guide to where in the HER-2/neu sequence potential $CD8^+$ T cell epitopes are located, once a specific peptide sequence is identified, more sensitive CTL assays can be done.

Following the determination that each of the vaccine constructs was capable of eliciting an anti-HER-2/neu immune response in a CTL assay, a panel of 3T3 cells expressing fragments of HER-2/neu (26) were used as target cells for vaccinations with each of the corresponding rLm vaccines (Table I). Each vaccine is capable of eliciting a CTL response to at least one fragment of HER-2/neu. Several fragments of HER-2/neu likely contain a subdominant epitope as evidenced by lysis that was significantly different from controls ($p < 0.05$). Based on the CTL analysis of this panel of target cells, regions of HER-2/neu with potential subdominant epitopes for the FVB/N mouse have been identified.

Fusion to LLO and delivery by L. monocytogenes *enhances the immunogenicity of HER-2/neu*

There are several possibilities for why these recombinant *L. monocytogenes* vaccines are capable of revealing subdominant epitopes when other vaccination strategies have not. The Lm-LLO-HER-2/neu vaccines may be revealing these subdominant epitopes through 1) delivery by *L. monocytogenes*, 2) fusion of the target tumor Ag to LLO, 3) breaking HER-2/neu into fragments, or 4) a combination of these factors. To determine which of these factors contributes to the enhanced immunogenicity of HER-2/neu in the Lm-LLO-HER-2/neu system, a series of DNA vaccines were constructed. Only the EC1 fragment was made to study the effect of breaking HER-2/neu into pieces because each Lm-LLO-HER-2/neu vaccine worked equally well in tumor regression studies. These vaccines consist of a pcDNA 3.1 backbone containing one of the following inserts: full-length HER-2/neu (pcDNA neu), full-length HER-2/neu fused to LLO (pcDNA LLO-neu), the EC1 fragment (pcDNA EC1), or the EC1 fragment fused to LLO (pcDNA LLO-EC1).

Following the construction of the pcDNA-HER-2/neu vaccines, a tumor regression experiment was done comparing these constructs with Lm-LLO-EC1. Mice were given 7×10^5 NT-2 cells s.c. and were vaccinated i.m. with the DNA vaccines plus GM-CSF on a separate pcDNA 3.1 plasmid and i.p. with Lm-LLO-EC1 or PBS on days 3, 10, and 18. GM-CSF was included as an adjuvant for the DNA vaccines because of its ability to enhance the efficacy of DNA vaccines (29). The best vaccine based on this experiment was Lm-LLO-EC1 (Fig. 6*A*). Two Lm-LLO-EC1-vaccinated mice never developed tumors and two more later regressed their tumors compared with the pcDNA LLO-EC1 group in which one mouse never developed a tumor. By day 62, the differences in tumor sizes of the Lm-LLO-EC1 and pcDNA LLO-EC1 groups are statistically different ($p < 0.05$) and remain so on day 76 by which time there are four mice in the Lm-LLO-EC1 group without

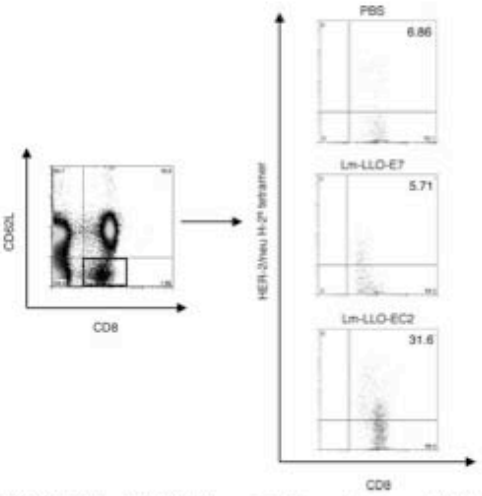

FIGURE 4. Lm-LLO-EC2 induces a 3-fold increase in tetramer$^+$,CD8$^+$ T cells. FVB/N mice were immunized and boosted 21 days later with 0.1 LD_{50} Lm-LLO-EC2 and Lm-LLO-E7 or 200 μl of PBS. Five days after the boost, splenocytes were stained with an H-2^d HER-2/neu tetramer, anti-CD8, and anti-CD62L.

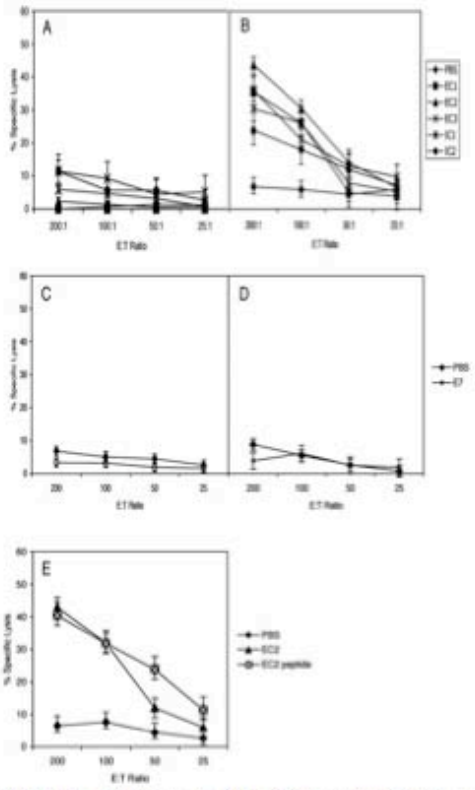

FIGURE 5. Each of the Lm-LLO-HER-2/neu vaccine constructs induces similar levels of anti-HER-2/neu CTL activity. FVB/N mice were immunized with 0.1 LD_{50} Lm-LLO-EC1, Lm-LLO-EC2, Lm-LLO-EC3, Lm-LLO-IC1, and Lm-LLO-IC2. Splenocytes were harvested 9 days later and cultured with irradiated NT-2 cells for 4 days. Following this restimulation, CTL activity was measured in a 4-h ^{51}Cr release from wild-type 3T3 (*A*) or 3T3-neu (full-length HER-2/neu) (*B*) targets. The ability of splenocytes from PBS-vaccinated mice was compared with splenocytes from Lm-LLO-E7 mice with either wild-type 3T3 (*C*) or 3T3-neu (*D*) target cells. *E*, Maximal levels of lysis in this type of assay was determined by the use of wild-type 3T3 target cells pulsed with the H-2^d-specific peptide. Results are shown as the mean of triplicate cultures. These results are representative of five experiments.

tumors, compared with only one mouse in the pcDNA LLO-EC1 group. Fusion to LLO was also compared through pcDNA EC1 vs pcDNA LLO-EC1 and pcDNA neu vs pcDNA LLO-neu (Fig. 6, *B* and *C*). The constructs where either the full-length HER-2/neu or the EC1 fragment are fused to LLO are clearly better in terms of tumor regression as compared with the unfused versions. Mice vaccinated with either of the unfused vaccines developed tumors that grew at the same rate as those in control mice vaccinated with just GM-CSF, which grew slightly slower than the tumors in mice vaccinated with only PBS. These differences in tumor sizes for pcDNA EC1 vs pcDNA LLO-EC1 and pcDNA neu vs pcDNA LLO-neu vaccinated mice are statistically significant ($p < 0.01$) on day 62 posttumor inoculation. The effect of breaking HER-2/neu up into smaller pieces was tested by comparing pcDNA LLO-neu vs pcDNA LLO-EC1 and pcDNA neu vs pcDNA EC1 (Fig. 6, *D* and *E*). Although there does seem to be minor differences in the tumor sizes between pcDNA LLO-EC1 and pcDNA LLO-neu between days 62 and 90 (Fig. 6*D*), this difference is not statistically significant. No difference could be seen between the pcDNA neu and pcDNA EC1-vaccinated mice, and tumors in both of these groups grew out as rapidly as the GM-CSF controls (Fig. 6*E*).

Broad CTL response to LLO-fused DNA vaccines vs unfused DNA vaccines

Each of the DNA vaccines was further analyzed using the standard ^{51}Cr release assay as described above. The LLO-fused versions of either full-length HER-2/neu or the EC1 fragment were capable of inducing T cells that lysed more of the 3T3-neu target cell lines than did the non-LLO-fused DNA vaccines (Table II). pcDNA neu induced a strong CTL response against only the 3T3-neu-4 target cell line, the line that contains the H-2^d epitope previously described by Ercolini et al. (26). The fusion of the full-length HER-2/neu to LLO resulted in a broadening of the regions that could be recognized by CTLs, and the same result was seen with the EC1 fragment fused to LLO vs the non-LLO-fused EC1. Thus, whereby only 3T3-neu-1 and 3T3-neu-4 were targeted for lysis by pcDNA neu splenocytes, 3T3-neu-1, -4, -5, and -9 were targeted by splenocytes from pcDNA-LLO-neu.

Discussion

Fusion of fragments of HER-2/neu to a truncated form of the *L. monocytogenes* hemolysin, LLO, and the delivery of the fusion constructs by *L. monocytogenes* increased the immunogenicity of this self-Ag. Five vaccines were constructed containing overlapping fragments of the rat HER-2/neu gene fused to a membrane inactive form of LLO. Based on previous epitope mapping, only

Table I. *Regions of HER-2/neu with potential H-2^d epitopes based on percent-specific lysis from CTL analysis and the corresponding vaccines*

Listeria Strain	Neu Region Spanned	Percent-Specific	Lysis of Target Cells* 200:1	100:1	Neu Regions Containing an Epitope
Lm-LLO-EC1	20–326	3T3-neu-1	14.3*	0.7	20–148
		3T3-neu-2	0	0	
		3T3-neu-3	6.5*	3.5	291–326
Lm-LLO-EC2	303–501	3T3-neu-3	10.9*	7.4	303–426
		3T3-neu-4	23.8*	8.4*	410–501
Lm-LLO-EC3	479–655	3T3-neu-4	1	0	
		3T3-neu-5	34.4*	25.3*	531–655
Lm-LLO-IC1	690–1081	3T3-neu-6	6.9*	9*	690–797
		3T3-neu-7	0	2.3	
		3T3-neu-8	18.2*	6.4	952–1081
Lm-LLO-IC2	1020–1260	3T3-neu-8	10.3*	8.2*	1020–1085
		3T3-neu-9	16.5	0	1063–1260

* Lysis of target cells shown as percent lysis minus background lysis by PBS control vaccinated mice for the E:T ratios of 200:1 and 100:1. Each well was set up in triplicate, and the results of a representative experiment are shown. * denotes statistically significant lysis above background level at $p < 0.05$. Percent-specific lysis before subtracting background PBS lysis was calculated as percent-specific lysis = 100 ((experimental lysis − spontaneous lysis)/(total lysis − spontaneous lysis)).

the Lm-LLO-EC2 vaccine was expected to generate an anti-HER-2/neu tumor response because this vaccine contains the only previously described rat HER-2/neu epitope for the FVB/N mouse, although there is evidence that this may not be the immunodominant epitope in the HER-2/neu-transgenic mouse (26, 30). Surprisingly, each of the five vaccine constructs generated an antitumor immune response that resulted in stasis of tumor growth and eventually a late onset of regression in a subset of the vaccinated mice. The antitumor immunity observed absolutely required CD8$^+$ T cells. The fact that each of these vaccine constructs could generate HER-2/neu-specific CD8$^+$ T cells and an antitumor response suggests that subdominant MHC class I-restricted HER-2/neu epitopes are being revealed through the use of these vaccines. This study shows that these epitopes emerge not only through the use of *L. monocytogenes* as a vaccine vector but also through the fusion of the HER-2/neu fragments to LLO.

Fusion to LLO was originally performed to ensure that the target Ag would be secreted by recombinant *L. monocytogenes*, resulting in presentation of the secreted target Ag by the MHC class I pathway and the generation of a CD8$^+$ T cell response (17). However, subsequent to these earlier experiments, we found that fusion of target Ags to either LLO or a PEST-like sequence contained in LLO resulted in an increased antitumor response (31). The exact reason for this enhancement in immunogenicity of fused Ags is not yet clear, but we hypothesize that these fused Ags are more readily degraded by the proteasome and subsequently targeted to the class I pathway. Decatur and Portnoy (32) have shown that deletion of a PEST-like sequence toward the N terminus of LLO results in an accumulation of LLO in the cytosol of the host cells, which suggests that this sequence may target LLO for degradation. However, we believe that LLO is doing more to increase the immunogenicity of target Ags than simply increasing the degradation and subsequent presentation of these Ags. We compared vaccines where the tumor Ag is fused to LLO vs fusion to PEST and showed that both elicited similar antitumor immune responses (31). However, even fusing the Ag to a LLO molecule from which the PEST sequence has been deleted resulted in better antitumor efficacy than using the Ag alone, suggesting that LLO may have an adjuvant effect quite apart from enhancing Ag processing. We have shown previously using vaccines that deliver the HPV-16 E7 Ag that Lm-LLO-E7 can mature bone marrow-derived dendritic cells and up-regulate costimulatory molecules, whereas Lm-E7 does not, which also points to an adjuvant effect for LLO (33).

Potential regions of HER-2/neu with subdominant epitopes have been identified through CTL analysis following vaccination of mice with DNA vaccines consisting of either full-length or the EC1 fragment of HER-2/neu both fused to LLO and unfused. Lysis of regions of HER-2/neu not containing the identified dominant epitope (26) was seen with each of the four DNA vaccines. A broadening of the regions of HER-2/neu with potential epitopes based on this CTL analysis can be observed with fusion to LLO, further solidifying the theory that LLO enhances the immunogenicity of target Ags leading to the revelation of subdominant epitopes. Comparing the DNA vaccines, it is clear that fusion of the full-length neu to LLO increases the regions of neu that are recognized from two, with no LLO fusion, to four with LLO fusion (Table II). However, this expanded response was not equal to the responses seen with the *Listeria*-based HER-2/neu vaccines, as a total of nine regions with potential epitopes have been identified with these vaccines (Table I). Furthermore, the comparison of tumor regression induced by pcDNA LLO-EC1 with that of Lm-LLO-EC1 shows that Lm-LLO-EC1 is superior to pcDNA LLO-EC1 (Fig. 6*A*). Therefore, the increased epitope recognition of the *Listeria*-based vaccines vs the DNA vaccines is likely due to enhancement of the magnitude of the immune response through delivery by *Listeria*, which enhances the detection level of weak epitopes. These DNA vaccine results suggest that some epitopes, but not all, can be revealed through fusion to LLO, but some need the additional boost of delivery by *L. monocytogenes* as well, perhaps because of the added danger signals provided by a live vaccine vector (34) in addition to direct delivery to the MHC class I pathway of Ag processing by professional APCs.

DNA-based vaccines targeting HER-2/neu have been evaluated by other groups in transplantable tumor models (35–38). A major difference between the Lm-LLO- or DNA-LLO-HER-2/neu vaccine studies described here and those performed by other groups is that all of our vaccines were tested in a therapeutic, rather than a prophylactic, mode. In our studies, the first DNA vaccination was given 3 days after injection of the tumor but before the appearance of a palpable tumor. In the pcDNA LLO-EC1 group, one mouse never developed a tumor and one mouse later rejected its tumor, whereas there was a slow growth of tumor in the pcDNA LLO-neu

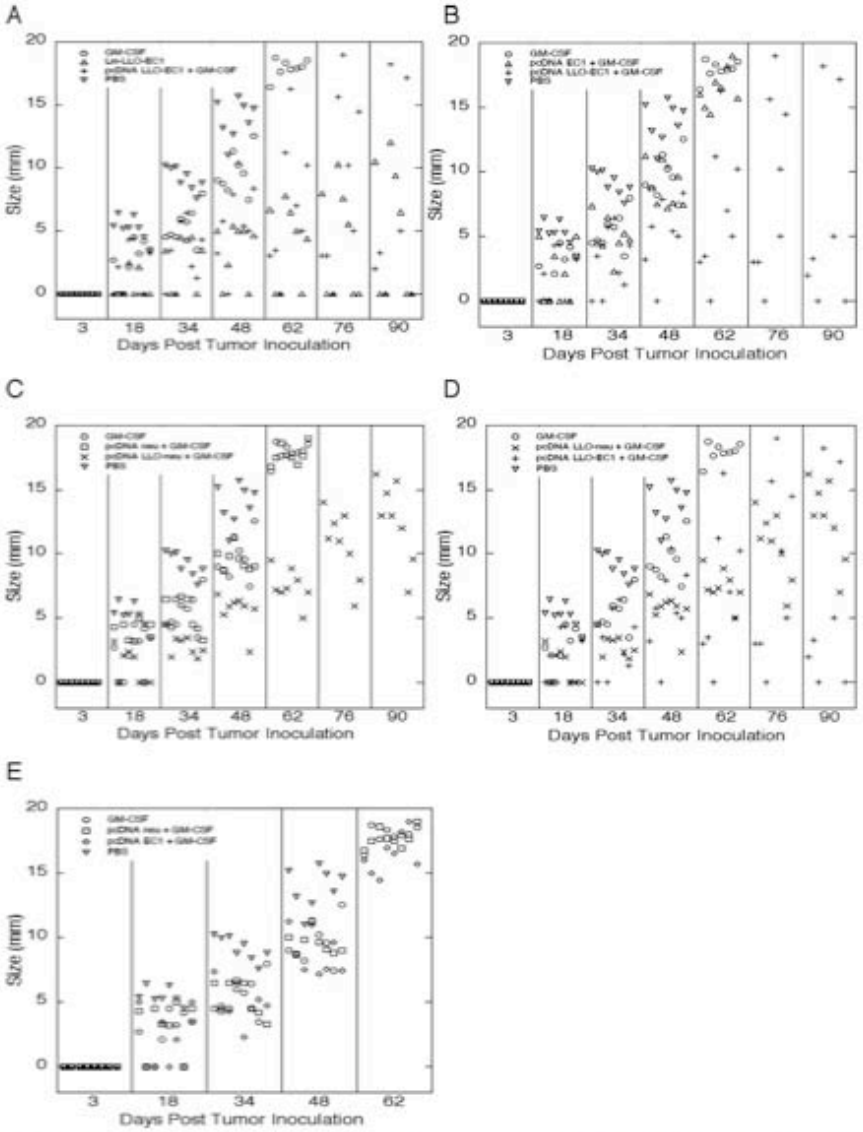

FIGURE 6. Delivery by *L. monocytogenes* and fusion to LLO increases the antitumor immune response of HER-2/neu vaccines. FVB/N mice (eight per group) received 7×10^5 NT-2 cells by s.c. injection in the right flank. Mice were treated on days 3, 10, and 18 with 0.1 LD_{50} Lm-LLO-EC1 or 50 μg of pcDNA neu + GM-CSF, pcDNA LLO-neu + GM-CSF, pcDNA EC1 + GM-CSF, pcDNA LLO-EC1 + GM-CSF, GM-CSF alone, or PBS. The average tumor diameter was measured with calipers and is shown for each mouse. Mice were sacrificed when tumor diameter reached ~2.0 cm, and tumor measurements are only shown for the surviving mice at each time point. Lm-LLO-EC1 vs pcDNA LLO-EC1 + GM-CSF (*A*), pcDNA EC1 + GM-CSF vs pcDNA LLO-EC1 + GM-CSF (*B*), pcDNA neu + GM-CSF vs pcDNA LLO-neu + GM-CSF (*C*), pcDNA LLO-neu + GM-CSF vs pcDNA LLO-EC1 + GM-CSF (*D*), and pcDNA neu + GM-CSF vs pcDNA EC1 + GM-CSF (*E*).

group (Fig. 6*D*). The Lm-LLO-HER-2/neu vaccines are given 7 days posttumor injection, at which point there is a 4- to 5-mm macroscopic tumor present. A subset of mice vaccinated with these vaccines do completely regress their tumors, which is a very important result because it is extremely difficult to cause the regression or induce a halt in the growth of established tumors. The

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Table II. *Regions of HER-2/neu with potential epitopes based on DNA vaccinations of wt FVB mice*

Listeria Strain	Neu Region Spanned	Percent-Specific	Lysis of Target Cells[a] 200:1	100:1	Neu Regions Containing an Epitope
peDNA neu + GM-CSF	1–1260	3T3-neu-1	17.4*	12.4*	20–148
		3T3-neu-2	0	0.1	
		3T3-neu-3	0	1.6	
		3T3-neu-4	23.4*	19.5*	410–479
		3T3-neu-5	0	0.3	
		3T3-neu-6	2.6	0	
		3T3-neu-7	0	0.6	
		3T3-neu-8	0	1.2	
		3T3-neu-9	0	0.1	
peDNA LLO-neu + GM-CSF	1–1260	3T3-neu-1	30.9*	20.5*	20–148
		3T3-neu-2	0.4	0	
		3T3-neu-3	1.9	1.7	
		3T3-neu-4	31.2*	25.5*	410–501
		3T3-neu-5	6.4*	6.4	479–531
		3T3-neu-6	0	0	
		3T3-neu-7	0	2.5	
		3T3-neu-8	0	4	
		3T3-neu-9	21*	15.8*	1085–1260
peDNA EC1 + GM-CSF	1–326	3T3-neu-1	7.8	1.9	
		3T3-neu-2	0	2.1	
		3T3-neu-3	8.5*	0	291–326
peDNA LLO-EC1 + GM-CSF	1–326	3T3-neu-1	14.6*	8.5*	20–148
		3T3-neu-2	0	0.5	
		3T3-neu-3	9.9*	5.1	291–326

[a] Lysis shown of each of the identified cell lines is identified, and lysis is noted as percent lysis minus background lysis from PBS control vaccinated mice. * denotes significant lysis above background at $p < 0.05$. Percent-specific lysis before the subtracting background levels was calculated as percent-specific lysis = 100 ((experimental lysis − spontaneous lysis)/(total lysis − spontaneous lysis)).

majority of HER-2/neu DNA vaccines produced by other groups were used in the less stringent prophylactic mode to determine whether vaccination will lead to protection against a subsequent tumor challenge (35–38). In addition, the majority of these studies have been performed in a BALB/c HER-2/neu tumor model, which shows a vastly different disease progression than the FVB/N mouse (36–38). Prophylactic DNA vaccination in the FVB/N mouse has not been shown to induce a complete rejection of a subsequent tumor challenge but will protect 25–100% of animals from developing tumors depending on the treatment used (35). In this study, the DNA vaccines studied were comprised of the extracellular domain of HER-2/neu, the transmembrane domain of HER-2/neu, or the intracellular domain of HER-2/neu. The extracellular domain DNA vaccine protected 25–35% of mice and that percentage was increased to 60–75% with the addition of IL-12. Approximately 50% of the mice were protected from tumor challenge after treatment with the intracellular DNA vaccine, and the administration of IL-12 increased this percentage of protected animals to between 80 and 100%. Sixty to 75% of mice were protected from tumor challenge with the administration of the transmembrane DNA vaccine, and this percentage remained the same with the addition of IL-12 (35).

In our study, each of the vaccines resulted in the stasis of tumor growth immediately following the first vaccination (Fig. 2). However, none of the tumors decreased in size until >1 mo following the last vaccination. Ag loss or mutation and the down-regulation of MHC class I molecules frequently result in the lack of tumor regression (39). In the case of HER-2/neu-overexpressing tumors, where the overexpression of the tumor Ag contributes to the transformed phenotype of the tumors, the loss of the tumor Ag could also result in a lack of tumor growth. Neu-negative variants have been observed after anti-HER-2/neu therapies (40). We are currently attempting to determine whether the tumors that do not decrease in size or undergo regression in response to the *Listeria*-based vaccines are down-regulating HER-2/neu.

The induction of tumor regression at >1 mo after the final boost is in contrast with our observations using other tumor Ags where tumor regression will often begin after the first immunization and is completed within 28 days (19, 21, 31). The delay in tumor regression observed with the Lm-LLO-HER-2/neu vaccines is not likely due to the reactivation of a memory response. Although, it is likely that memory cells are generated following the recombinant *L. monocytogenes* vaccines, no additional vaccination or stimulus is given to reactivate these memory cells at the time that the tumor regression begins. We think it is more plausible that the increase in antitumor reactivity at a delayed time point is due to epitope spreading by cross-presentation of tumor-derived HER-2/neu or other Ags.

Epitope spreading has been seen previously in many different tumor models and can result in the generation of a specific antitumor response to cryptic and subdominant epitopes that were not initially part of the treatment administered (41–43). The *Listeria*-based vaccines containing fragments of HER-2/neu may be generating sufficient initial antitumor $CD8^+$ T cells to limit the growth of the tumor early after vaccination. Although the tumor growth is stabilized with new tumor cells being generated at the same rate that tumor cells are dying, APCs such as dendritic cells may be acquiring pieces of the dying cells, traveling to the draining lymph nodes, and cross-presenting these Ags to activate a much broader $CD8^+$ T cell response than could be generated by vaccination targeting one region of the HER-2/neu molecule. This phenomenon has been observed previously, and cross-presentation by $CD11c^+$ dendritic cells is seen as a key factor in the control of tumor growth because the $CD8^+$ T cell response elicited by a specific immunotherapy can be significantly enhanced by this mechanism (44, 45). We intend to explore this hypothesis by testing the reactivity of the

lymphocytes in the tumor-draining lymph nodes to see whether T cells specific to a region of HER-2/neu that was not included in that particular vaccine construct emerges at the time that regression begins. However, this task will be easier once we have completed our epitope mapping of the entire HER-2/neu molecule, which is currently underway. We have already determined that there are a large number of $CD11c^+$ dendritic cells infiltrating the tumors following the vaccinations before the onset of regression (data not shown) that could cross-present Ags.

The most effective antitumor response will likely be generated by enhancing low-affinity anti-HER-2/neu-specific $CD8^+$ T cells that were not deleted during thymic selection (46). This will be particularly imporiant in transgenic mouse models of cancer in which the mice are tolerant to the tumor Ag and the high-affinity T cells that can be generated to target the tumor in wild-type (non-tolerant) mice will not be present (47, 48). Makki et al. (49) and Kedl et al. (50) have shown that targeting an immunodominant tumor epitope can fail to result in a strong enough antitumor response that will result in the eradication of a tumor. Anti-HER-2/neu therapies that can lead to the amplification of subdominant epitopes are likely be more effective than vaccination against dominant epitopes.

Potentially confounding the efficacy of these vaccines is the fact that the vaccines are based on the rat HER-2/neu gene but that these vaccines are being tested in mice. In this study, the wild-type FVB/N mice are not specifically tolerant to the rat HER-2/neu gene, although the mouse and rat HER-2/neu genes are >90% homologous (51), so some degree of tolerance is expected. Although these sequences are extremely homologous, there are differences in the degree of homology for the different vaccine constructs. The IC1 construct containing the highly conserved kinase domain is 98% homologous to the mouse protein sequence, and this sequence shows the highest level of homology among the five vaccine constructs. Both the EC1 and EC2 regions are 94% homologous with the corresponding mouse regions, and the EC3 and IC2 regions are 93% homologous. In all of the vaccines, excluding Lm-LLO-IC1, the amino acid differences are spread throughout the entire fragment, whereas in IC1 the differences flank the kinase domain with only one nonhomologous amino acid in the kinase domain making the kinase domain 99.6% homologous. Interestingly, the CTL analysis of the *Listeria*-based vaccines (Table I) shows that of the three 3T3 lines that can be targeted by this fragment only two, 3T3-neu-6 and 3T3-neu-8, were capable of being lysed by effector cells induced by Lm-LLO-IC1 vaccination. Although we do not yet know the exact sequences of the MHC class I epitopes that are recognized in these fragments, it is possible that there are no epitopes present in the kinase domain, as this region is almost entirely homologous between the mouse and the rat. In addition, the portion of HER-2/neu contained in 3T3-neu-7 falls entirely in the kinase domain, and there is no CTL response against this region.

We do not observe a strong correlation between CTL activity and tetramer analysis of HER-2/neu-specific T cells from mice immunized with Lm-LLO-EC2, for which we have a tetramer available. This is because for most of the vaccines, the only source of Ag available to stimulate bulk populations of T cells in splenocytes is irradiated tumor cells. The NT-2 cells do not express a high level of Ag and thus are not very potent stimulators of effector cells. Furthermore, the effector cells used are a polyclonal T cell population that is derived from whole splenocytes, so $CD8^+$ T cells are not the only cells being counted as effector cells. In contrast, tetramer-positive T cells can be analyzed in the activated $CD8^+$ T cell population. Despite these limits to the CTL assay, we do see statistically significant levels of killing of the target cells from which we are able to narrow down potential areas that contain CTL epitopes.

We have shown in this study that vaccines generated from fragments of HER-2/neu that do not contain the dominant epitope are as effective as the vaccine that does result in $CD8^+$ T cells specific to the dominant epitope when fused to LLO and delivered by *L. monocytogenes*. Work is now underway to identify these subdominant epitopes and to test these vaccines in a HER-2/neu-transgenic mouse model for breast cancer (30).

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Disclosures

Y. Paterson has a financial interest in Advaxis, a vaccine and immunotherapeutic company that is developing *L. monocytogenes* as a cancer vaccine vector.

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